High LET Radiation Amplifies Centrosome Overduplication Through a Pathway of γ-Tubulin Monoubiquitination.

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CITATION:

Shimada, Mikio ...[et al]. High LET Radiation Amplifies Centrosome Overduplication Through a Pathway of γ-Tubulin Monoubiquitination.. International journal of radiation oncology, biology, physics 2013, 86(2): 358-365

ISSUE DATE:

2013-06-01

URL:

http://hdl.handle.net/2433/174323

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Abstract

Purpose: Radiation induces centrosome overduplication leading to mitotic catastrophe and tumorigenesis. Since mitotic catastrophe is one of the major tumor cell killing factors in high LET radiation therapy and long-term survivors from such treatment have a potential risk of secondary tumors, we investigated here LET-dependence of radiation-induced centrosome overduplication and the underlying mechanism.

Methods: Carbon and iron ion beams (13 - 200 keV/μm) and γ-rays (0.5 keV/μm) were used as radiation sources. To count centrosomes after IR exposure, human U2OS and mouse NIH3T3 cells were immunostained with antibodies of γ-tubulin and centrin 2. Similarly, *Nbs1*-, *Brca1*-, *Ku70*-, *DNA-PKcs*- deficient mouse cells and their counterpart wild type cells were used for measurement of centrosome overduplication.

Results: The number of excess centrosomes-containing cells at interphase and the resulting multipolar spindle at mitosis were amplified with increased LET and reached a maximum level at 100 keV/μm, then followed by sharp decrease in frequency. Interestingly, *Ku70* and *DNA-PKcs* deficiencies marginally affected the induction of centrosome overduplication, while the cell killings were significantly enhanced. This was in contrast to observation that high LET radiation significantly enhanced frequencies of centrosome overduplication in *Nbs1*- and *Brca1*-deficient cells. Since NBS1/BRCA1 is implicated in monoubiquitination of γ-tubulin, subsequently we tested if it is affected by high LET radiation. As a result, monoubiquitination of γ-tubulin was abolished in 48-72 hrs after exposure to high LET radiation, although γ-ray exposure slightly decreased it 48 hrs post-irradiation and it was restored to a normal level at 72 hrs.

Conclusion: High LET radiation significantly reduces NBS1/BRCA1-mediated monoubiquitination of γ-tubulin and amplifies centrosome overduplication with a peak at 100 keV/μm. On the other hands, *Ku70* and *DNA-PKcs* deficiencies mitigate centrosome overduplication, although deficiencies of both *NBS1/BRCA1* and *Ku70*/*DNA-PKcs* markedly enhance their cell killings.

Key words: centrosomes, ion beam, LET, NBS1, BRCA1, Ku70, DNA-PKcs, γ-tubulin, monoubiquitination

Introduction

Centrosomes, organelles consisting of centrioles and pericentriolar material, function as microtubule organizing center during mitosis (1). Centrosomes duplicate only once during each cell division, to provide two centrosomes, which form a bipolar mitotic spindle. Improper duplication of centrosomes results in excess or supernumerary centrosomes and forms multipolar spindles, leading to cell killing through mitotic catastrophe (2, 3). It has been reported by others that a small fraction of excess centrosome-containing cells can survive and forms polyploidy clonogenic cells, which are associated with tumorigenesis and tumor progression (4). Indeed, excess centrosomes have been frequently observed in many tumors (5).

BRCA1, a protein responsible for familial breast cancer, is an E3 ubiquitin ligase, which regulates DNA repair and cell cycle checkpoint (6). BRCA1 is directly involved in maintenance of centrosomes duplication through monoubiquitination of γ-tubulin, main component of centrosomes, at positions of K48 and K348 (7). XX have previously shown that centrosome overduplication frequently occurs in cells from patients with Nijmegen breakage syndrome (NBS), which is characterized by high sensitivity to radiation and predisposition to tumors (8). NBS1, the protein responsible for NBS, interacts with BRCA1 in centrosomes and regulates BRCA1-dependent centrosome duplication. Depletion of either BRCA1 or NBS1 with siRNA results in centrosome overduplication through decreased monoubiquitination of γ-tubulin (8).

High linear energy transfer (LET) radiation, such as carbon ion and iron ion beams, has beneficial physical and biological aspects that improve radiotherapy (9). Bragg peak from heavy-ion beams induces regional damage to tumor without exacerbation of normal tissue complications. High LET radiation produces densely ionization along their trajectories and incurs complex and clustered DNA damage that provides high biological effects with a peak at 100 keV/μm (10). High LET radiation therapy is clinically applied worldwide including the Heavy-Ion Medical Accelerator (HIMAC) in Chiba, Japan (9). Although high LET radiation therapy alone has provided favorable clinical outcome, interest is increasing as combined modalities with anticancer drug and gene therapies (11). Moreover, late effects, such as secondary tumor induction, are gradually becoming a matter of concern (12), and

hence, it is important to understand the mechanism of biological effect by high LET radiation.

Radiation-induced cell killing is affected by several factors including efficiency of DNA repair, chromosome aberration and apoptosis activity (13, 14). Mitotic catastrophe is implicated as a key factor of high LET radiation-induced cell killing. Ianzini et al. reported mitotic catastrophe in 35% of cells irradiated with 1 Gy of 31 keV/μm proton beams (15). On the other hands, Sudo et al. showed that high LET radiation can induce supernumerary centrosome formation in finite life-span human mammary epithelial cells (16), which is associated with mitotic catastrophe and is also a potential factor of tumorigenesis (2, 3, 17). However, LET dependence of centrosome overduplication remains to be determined. We investigated here LET dependency of centrosome overduplication by using carbon ion and iron ion beams generated from HIMAC, and also a role of γ-tubulin monoubiquitination in their inductions using DNA repair-deficient cell lines.

Material and Methods

Cell Culture

The human U2OS and the mouse NIH3T3 cells were used as wild-type cell lines. A31-1 (*Nbs1*-/-) MEF, the A31-1 MEF complemented with *NBS1* cDNA, *Brca1*-/- mouse ES cells, *Brca1*+/- mouse ES cells, mouse SCID (*DNA-PKcs*-/-) cells, the parental CB-17 (*DNA-PKcs*+/+) cells, *Ku70*-/- MEF and the *Ku70*-/- MEF complemented with *Ku70* cDNA, were obtained as previously reported (18). Cells were cultured with DMEM containing 10% fetal bovine serum (FBS).

Irradiation

Cells were irradiated with ^{137}Cs γ-rays (0.5 keV/μm) at a dose rate of 1Gy/min using a Gammacell 40 system (MDS Nordion). Carbon-ion (13 keV/μm, 50 keV/μm, 70 keV/μm, 100 keV/μm) and iron-ion beams (200 keV/μm) were generated by HIMAC at National Institute of Radiological Science in Chiba.

Cell survival assay

Cell survival was determined using colony formation assay. After exposure at indicated doses, cells were counted and plated on 100 mm dishes. 10-14 days later, cells were fixed with 100% ethanol and stained with crystal violet for counting colonies.

Immunostaining

Cells were cultured on slide glass (Matsunami) or slide chamber (Nunc) and irradiated with γ-rays or ion beams. 72 hrs after exposure, cells on coverslips were washed with phosphate-buffered saline (PBS), fixed in cold methanol (-20°C) for 10 min and incubated with a detergent solution (0.1% Triton-X100, 20 mM HEPES pH 7.4, 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose) at 4°C for 5 min. The cells were then incubated with 3% non-fat milk for 30 min, followed by incubation with primary antibodies against γ-tubulin (sc17787, Santa Cruz). Centrosomes were also stained with centrin-2 (sc27793R, Santa Cruz), to delineate it. The cells were subsequently incubated with the following secondary antibodies: Alexa-488-conjugated anti-rabbit IgG (Molecular Probes) for γ-tubulin and Alexa-546-conjugated anti-rabbit IgG (Molecular Probes) for centrin-2. Fluorescence was visualized using a confocal laser-scanning microscope (Leica). At least 200 cells in each sample were examined for centrosome number and nuclear morphology.

Western blotting

Cell extracts were prepared using RIPA buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.1% SDS, 1.0% TritonX-100, 0.1% Sodium deoxycholate, 1 mM EDTA, 1 × proteinase inhibitor, PMSF, aprotinin, leupeptin), or IP buffer (20 mM Hepes-NaOH at pH 7.4, 0.2% NP-40, 150 mM NaCl, 25% glycerol, 0.1 mM EDTA). The γ-tubulin was immunoprecipitated with Anti-γ-tubulin (T3559, Sigma Chemical Co) according to the method previously described (8). Western blotting was performed using anti-γ-tubulin (T3559, Sigma Chemical Co), FK2 anti-mono- and poly-ubiquitin (PW8810, Biomol international, LP). Anti-rabbit IgG (sc2027, Santa Cruz) was used as a negative control.

Statistical analysis

Values represented the mean and standard error (mean±SE) of three independent experiments. The differences between groups were determined by Student's *t*-test. A *p*-value less than 0.05 was selected as the criterion for a statistically significant difference.

Results

Centrosome overduplications were increased with high LET irradiation.

Since we previously showed induction of centrosome overduplication by irradiation with γ-rays (18), frequency of the γ-ray-induced centrosome overduplication was compared with that of cells irradiated with high LET radiation using carbon ion beam (13 keV/μm) and iron ion beam (200 keV/μm). The number of centrosomes in U2OS and NIH3T3 cells at interphase was counted 72 hrs after irradiation (Figure 1a). The frequencies of excess centrosome-containing U2OS cells were increased with graded dose and were enhanced with increased LET of radiation (Figure 1b). The frequency of excess centrosome-containing cells irradiated with 5 Gy of iron-ion beam was more than twice than that with 5 Gy of γ-rays. Similar results were obtained using NIH3T3 cells, although the frequencies of radiation-induced centrosome overduplication were higher in NIH3T3 cells than in U2OS cells (Figure 1c).

Multipolar spindles were increased with high LET irradiation.

Centrosome overduplication induces multipolar spindle, which leads to mitotic catastrophe (2, 3). Subsequently, we quantified the occurrence of multipolar spindles in U2OS cells at mitosis after exposure to γ-rays or high LET radiation. Formations of multipolar spindles in cells irradiated with 5 Gy of iron-ion (200 keV/μm) and carbon-ion (13 keV/μm) were 32% and 22%, respectively, which were significantly higher 12% of cells irradiated with 5 Gy of γ–rays (Figure 2a and 2b). Similarly, iron ion and carbon ion beams efficiently induced cell killings, in comparison with that of cells after γ–rays radiation (Supplementary Figure 1). To clarify LET dependency of multipolar spindle formation, we compared it using

carbon-ion beam with a wide range of LET (13, 50, 70, 100 keV/μm). The ratios of multipolar spindle cells after high LET radiation to that after γ–rays were increased with increased LET and, similar to radiation-induced cell killing, reached a maximum at 100 keV/μm, although that of radiation-induced cell killing at 100 keV/μm was higher than that of multipolar spindle (Figure 3). This difference might be due to the involvement of factors other than mitotic catastrophe in cell killing.

Radiation-induced centrosome overduplication in DNA repair-deficient cell lines.

We previously showed that centrosome overduplications are amplified by depletion of either Nbs1 or Brca1 (8). To investigate the involvement of DNA repair proteins on induction of centrosome overduplications after exposures to γ–rays and high LET radiation, we measured the radiation-induced centrosome overduplication in cells deficient with homologous recombination repair proteins including NBS1 and BRCA1, or with non-homologous end-joining proteins including Ku70 and DNA-PKcs. As a result, centrosome overduplications in *Nbs1*- or *Brca1*- deficient cells were increased with graded doses of γ–rays, and they were significantly higher than that in counterpart wild type cells (Figure 4a and 4b). Similar to centrosome overduplication, high LET radiation enhanced radiation-induced cell killing (Supplementary Figure 1). These enhancements of centrosome overduplication and cell killing by *Nbs1*- or *Brca1*- deficiency were also observed after exposures to high LET radiation with 13 keV/μm and 200 keV/μm. We next investigated the radiation-induced centrosome overduplications in *Ku70*- and *DNA-PKcs*-deficient cells. In contrast to *Nbs1*- or *Brca1*- deficient cells, centrosome overdupliation in *Ku70*-deficient and *DNA-PKcs*-deficient cells were at a level similar to that of counterpart wild type cells at each dose and LET of radiation (Figure 5a and 5b). However, enhanced cell killings were observed in either *Ku70*- or *DNA-PKcs*-deficient cells irradiated with γ–rays and 200 keV/μm iron ion beams, as observed in *Nbs1*- or *Brca1*- deficient cells.

Compromized monoubiquitination of γ–tubulin after exposure to high LET radiation.

Monoubiquitination of γ–tubulin regulates centrosome duplication, because the

decreased monoubiquitination of γ–tubulin, as observed in *Nbs1*- and *Brca1*-deficient cells, enhances centrosome duplication (8). We next investigated the monoubiquitination level of γ–tubulin in U2OS cells up to 120 hrs after irradiation. The cell lysates after irradiation were immunoprecipitated with γ–tubulin antibody and blotted using FK2 antibody. Although FK2 antibody recognizes both mono- and poly-ubiquitin after immunoprecipitated by γ–tubulin antibody, we observed a single band monoubiquitination of γ–tubulin, but not polyubiquitination. Monoubiquitination of γ–tubulin was slightly decreased 24-48 hrs after exposure to γ–rays, and it was restored to normal level 72 hrs later (Figure 6). On the other hand, iron ion beam radiation abolished the monoubiquitination of γ–tubulin 24-48 hrs post-irradiation and it was not restored even 120 hrs later. The decrease in monoubiquitination after irradiation with high LET was at the level similar to that of *Nbs1*-deficient cells (8).

Discussion

We showed here that high LET radiation efficiently induced centrosome overduplication with a maximum level at 100 keV/μm, which was similar to that of radiation-induced cell killing (Figure 3). Although deficiencies of all DNA repair proteins tested here enhanced cell killing, enhancements of centrosome overduplication were observed in *Nbs1*- and *Brca1*-deficient cells but not in *Ku70*- and *DNA-PKcs*-deficient cells (Figure 4 and 5). This could be because NBS1/BRCA1 proteins are involved in monoubiquitination of γ-tubulin, the disruption of which enhances centrosome overduplication (8). Indeed, γ-tubulin monoubiquitination was decreased more severely in cells irradiated with high LET than that with γ-rays (Figure 6). On the other hand, radiation-induced centrosome overduplications in *Ku70*- and *DNA-PKcs*-deficient cells might be contributed predominantly by cell cycle checkpoint-dependent pathway, rather than decreased monoubiquitination of γ-tubulin, as we previously reported (18).

Centrosome overduplication was converted to multipolar spindles with an efficiency of more than 90 % after exposure to either γ-rays or 200 keV/μm iron ion beam, as observed in Figure 2: fractions of multipolar spindles in U2OS cells, containing 15% or 38% of centrosome overduplication after exposure to either 5 Gy

of γ-rays or iron ion beam, were 12% or 35% at mitosis, respectively. The difference in ratios between centrosome overduplication and multipolar cell division might be due to failure of cytokinesis after irradiation (4). Although these multipolar spindle-containing cells may convert to mitotic catastrophe, there are additional factors derived from the other radiation damage. Using time-lapse microscopy for up to 70 hrs, Dodson reported that mitotic catastrophes after 10 Gy of γ-rays are induced in 50% of mitotic cells, of which approximately 70% is derived from centrosome overduplication. The remaining 30% could be due to other radiation damage, such as dicentric chromosome, because it was not observed in un-irradiated cells (3). On the other hand, a majority of these cells that undergo mitotic catastrophe must die and a small fraction could survive. Time-lapse imaging analysis for 266 hrs by others revealed that, among 210 multipolar division in HeLa cells irradiated with 10 Gy of γ-rays, only four cells with polyploidy can survive and restore the proliferative state of tumor cell population through depolyploidization, which recapitulates a process of tumor progression (4). Thus, high LET radiation, which efficiently causes the cell killing by mitotic catastrophe through radiation-induced centrosome overduplications, is a potential factor for tumor progression or secondary tumor development in cells surviving mitotic catastrophe.

It has been reported that monoubiquitination of γ–tubulin is required to maintain the proper number of centrosomes in cells (8). This pathway is mediated with NBS1 and BRCA1, and disruptions of these proteins cause the centrosome overduplication by decreased monoubiquitination. We showed here radiation also reduces the monoubiquitination of γ–tubulin (Figure 5). Lately, Falck et al reported that CDK phosphorylates NBS1 at position of S432, which is essential for activation of NBS1 including DNA-end resection and homologous recombination (19). Interestingly, this phosphorylation of NBS1 is gradually decreased after irradiation and is abolished 3 hrs later, suggesting the attenuation of γ–tubulin monoubiquitination after irradiation through inactivation of NBS1. This also might explain why centrosome overduplication is not enhanced in *Ku70*- and *DNA-PKcs*-deficient cells. Since *Ku*-deficiency enhances homologous recombination (20), NBS1 phosphorylation state continues for longer time in *Ku70*- and *DNA-PKcs*-deficient cells, and as a result, this persistent NBS1 activation could

counteract the attenuated monoubiquitination of γ–tubulin after irradiation. Therefore, inhibition of non-homologous end-joining is useful for both enhancement of cell killing and repression of the secondary tumor or tumor progression in high LET radiation therapies.

In summary, we firstly showed here that high LET radiation significantly reduces monoubiquitination of γ-tubulin and, thereby, amplifies centrosome overduplication, with a peak at 100 keV/μm, although centrosome overduplication might raise the possibility of tumorigenesis. The γ–tubulin monoubiquitination is mediated by NBS1 and BRCA1 and as a result, disruptions of NBS1 and BRCA1 amplified centrosome overduplication, while it was not affected by disruptions of *Ku70* and *DNA-PKcs*. This could provide useful information to decrease the possibility of tumorigenesis in high LET radiation therapies. These evidences suggest that adjuvant treatment to inhibit non-homologous end-joining, improve high LET radiation therapy without provocation of the secondary tumor development.

References

1. Shimada M, Komatsu K. Emerging connection between centrosome and DNA repair machinery. *J Radiat Res (Tokyo)* 2009;50:295-301.
2. Eriksson D, Lofroth PO, Johansson L, *et al.* Cell cycle disturbances and mitotic catastrophes in HeLa Hep2 cells following 2.5 to 10 Gy of ionizing radiation. *Clin Cancer Res* 2007;13:5501s-5508s.
3. Dodson H, Wheatley SP, Morrison CG. Involvement of centrosome amplification in radiation-induced mitotic catastrophe. *Cell Cycle* 2007;6:364-370.
4. Ianzini F, Kosmacek EA, Nelson ES, *et al.* Activation of meiosis-specific genes is associated with depolyploidization of human tumor cells following radiation-induced mitotic catastrophe. *Cancer Res* 2009;69:2296-2304.
5. Nigg EA. Centrosome aberrations: cause or consequence of cancer progression? *Nat Rev Cancer* 2002;2:815-825.
6. Huen MS, Sy SM, Chen J. BRCA1 and its toolbox for the maintenance of genome integrity. *Nat Rev Mol Cell Biol* 2010;11:138-148.
7. Sankaran S, Starita LM, Simons AM, *et al.* Identification of domains of BRCA1 critical for the ubiquitin-dependent inhibition of centrosome function. *Cancer Res* 2006;66:4100-4107.
8. Shimada M, Sagae R, Kobayashi J, *et al.* Inactivation of the Nijmegen breakage syndrome gene leads to excess centrosome duplication via the ATR/BRCA1 pathway. *Cancer Res* 2009;69:1768-1775.
9. Hamada N, Imaoka T, Masunaga S, *et al.* Recent advances in the biology of heavy-ion cancer therapy. *J Radiat Res (Tokyo)* 2010;51:365-383.
10. Franken NA, ten Cate R, Krawczyk PM, *et al*. Comparison of RBE values of high-LET α-particles for the induction of DNA-DSBs, chromosome aberrations and cell reproductive death. *Radiat Oncol* 2011;6:64.
11. Kitabayashi H, Shimada H, Yamada S, *et al.* Synergistic growth suppression induced in esophageal squamous cell carcinoma cells by combined treatment with docetaxel and heavy carbon-ion beam irradiation. *Oncol Rep* 2006;15:913-918.

12. Newhauser WD, Durante M. Assessing the risk of second malignancies after modern radiotherapy. *Nat Rev Cancer* 2011;11:438-448.
13. Asaithamby A, Hu B, Chen DJ. Unrepaired clustered DNA lesions induce chromosome breakage in human cells. *Proc Natl Acad Sci U S A* 2011;108:8293-8298.
14. Smith LE, Nagar S, Kim GJ*, et al.* Radiation-induced genomic instability: radiation quality and dose response. *Health Phys* 2003;85:23-29.
15. Ianzini F, Cherubini R, Mackey MA. Mitotic catastrophe induced by exposure of V79 Chinese hamster cells to low-energy protons. *Int J Radiat Biol* 1999;75:717-723.
16. Sudo H, Garbe J, Stampfer MR, *et al*. Karyotypic instability and centrosome aberrations in the progeny of fitnite life-span human mammary epithelial cells exposed to sparsely or densely ionizing radiation. *Radiat Res*;170:23-32.
17. Maxewll CA, Fleisch MC, Costes SV, *et al*. Targeted and nontargeted effects of ionizing radiation that impact genomic instability. *Cancer Res* 2008;68:8304-8311.
18. Shimada M, Kobayashi J, Hirayama R*, et al.* Differential role of repair proteins, BRCA1/NBS1 and Ku70/DNA-PKcs, in radiation-induced centrosome overduplication. *Cancer Sci* 2010;101:2531-2537.
19. Falck J, Forment JV, Coates J*, et al.* CDK targeting of NBS1 promotes DNA-end resection, replication restart and homologous recombination. *EMBO Rep* 2012;13:561-568.
20. Pierce AJ, Hu P, Han M*, et al.* Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells. *Genes Dev* 2001;15:3237-3242.

Figure legends

Figure. 1. Enhanced centrosome overduplications by exposure to high LET radiation. Immunostainings of U2OS cells at interphase with γ–tubulin antibody and centrin-2 antibody were performed 72 hrs after exposures to 5 Gy of γ–rays, carbon-ion beam and iron-ion beam (a). Cells with more than 2 centrosomes were counted in U2OS cells (b) and NIH3T3 cells (c) after exposures to γ–rays and ion beams at indicated doses. At least 200 cells were counted and examined 3 times, respectively.

Figure. 2. Enhanced multipolar spindles by exposure to high LET radiation. Immunostainings of U2OS cells at mitosis with γ–tubulin antibody and centrin-2 antibody were performed 72 hrs after exposures to 5 Gy of γ–rays, carbon-ion beam and iron-ion beam (a). Cells with multipolar spindle at mitosis were counted in U2OS cells after irradiation with 5 Gy of radiation with indicated LET (b). At least 200 cells were counted and examined 3 times, respectively. Student's t-test was performed between 70 keV/μm and 100 keV/μm, and indicated statistically significant increase ($p<0.05$).

Figure. 3. LET-dependent formation of radiation-induced multipolar spindle and cell killing. The enhancement ratios of multipolar spindle formation in cells irradiated with 5Gy of high LET radiation to that of γ–rays (0.5 keV/μm), as shown in Figure 2, were plotted with the LET. Similarly, the ratios of surviving fractions at 5Gy, as shown in Supplementary Figure, were plotted with the LET.

Figure 4. Radiation-induced centrosome overduplication in HR repair-deficient cell lines. The immunostainings were performed after irradiation, similar to Figure 1, and more than 2 centrosomes-containing cells were counted in A31-1 (*Nbs1*-deficient cells) and the cells complemented with wild type NBS1 cDNA (a), *Brca1* knockout mouse cells (*Brca1*-/-) and the heterozygote mouse cells (*Brca1*+/-) (b), At least 200 cells were counted and examined 3 times, respectively. Student's

t-test was performed after deduction of the frequency at 0 Gy from that at 5 Gy irradiation and indicated statistically significant increase ($p<0.05$). Similar statistical significances were obtained at doses of 1 Gy and 3 Gy.

Figure 5. Radiation-induced centrosome overduplication in NHEJ repair-deficient cell lines. The immunostainings were perforemd after irradiation, similar to Figure 1, and more than 2 centrosomes-containing cells were counted in *Ku70* knockout mouse cells (*Ku70*-/-) and the cells complemented with wild type Ku70 cDNA (a), SCID mouse cells (*DNA-PKcs*-/-) and the parental CB17 mouse cells (*DNA-PKcs*+/+) (b). At least 200 cells were counted and examined 3 times, respectively. Student's t-test was performed, similar to Figure 4, but indicated statistically no significant increase ($p>0.05$). No statistical significance was also observed at doses of 1 Gy and 3 Gy.

Figure. 6. Time course of γ–tubulin monoubiquitination after irradiation. U2OS cells were irradiated with γ-ray (0.5 keV/μm) (a) and iron ion beam (200 keV/μm) (b). Cell lysates were immunoprecipitated with γ–tubulin antibody at the indicated time after irradiation and blotted with FK2 antibody. Mouse IgG antibody was used as a negative control.

Figure 1

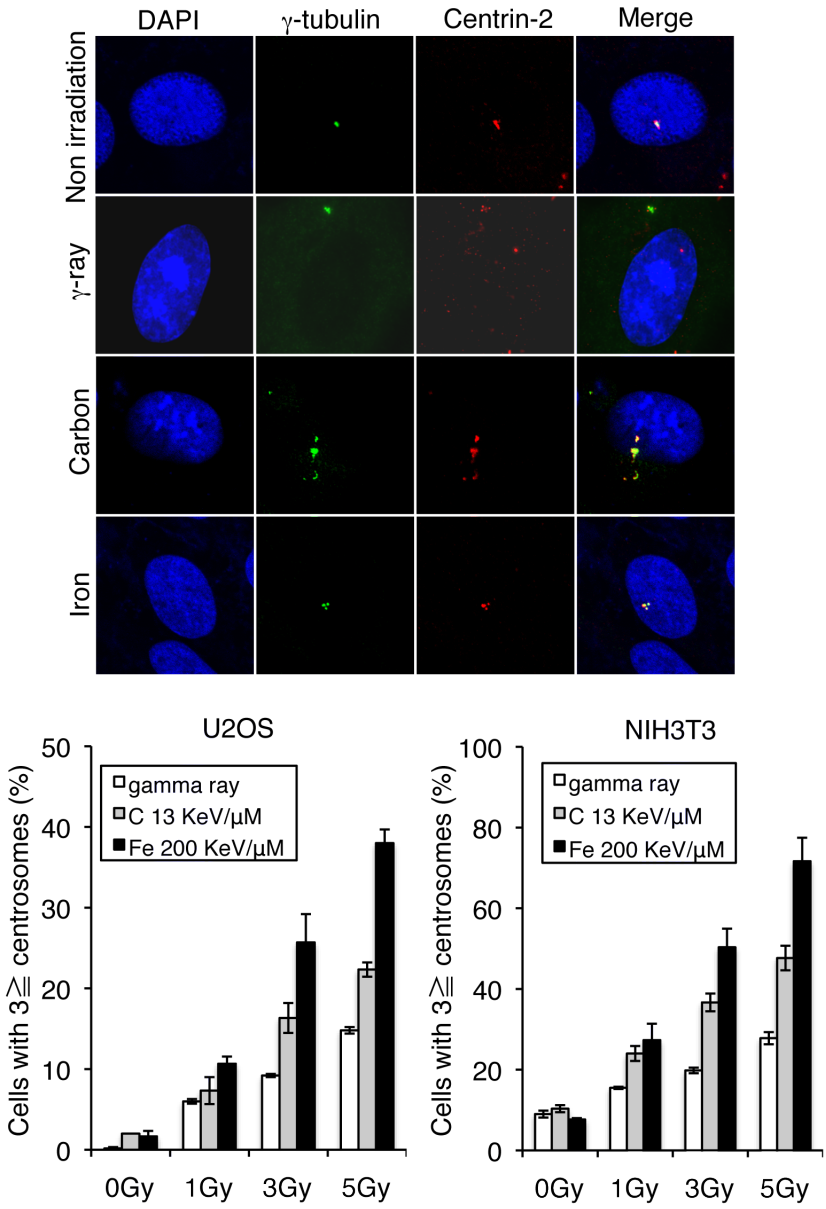
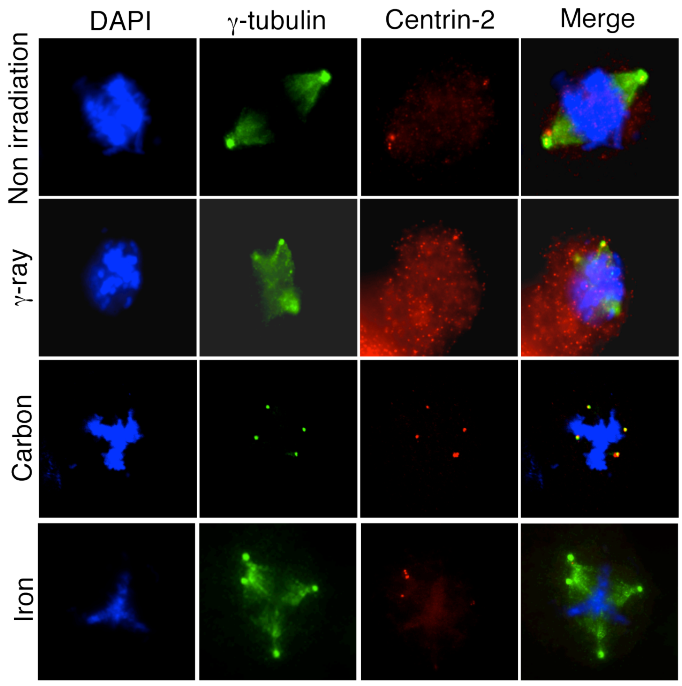


Figure 2



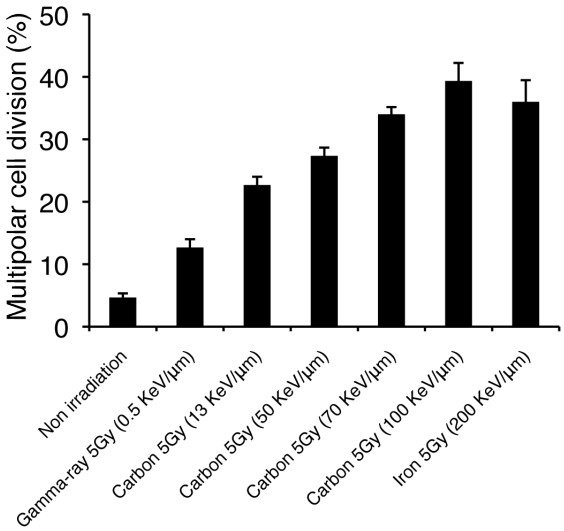


Figure 3

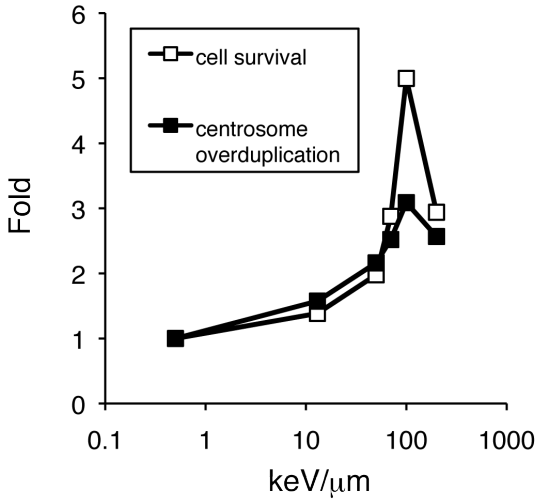


Figure 4

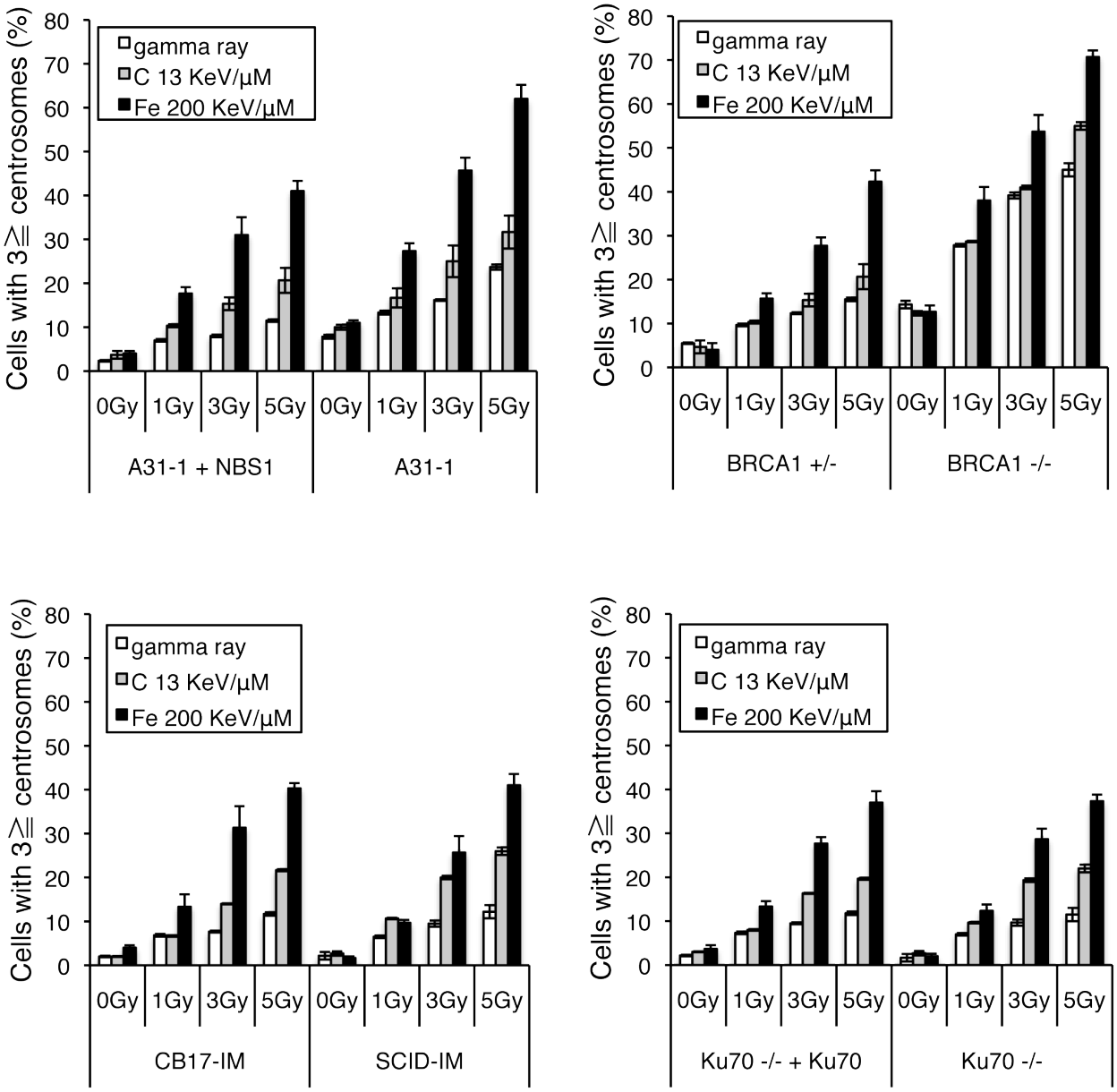
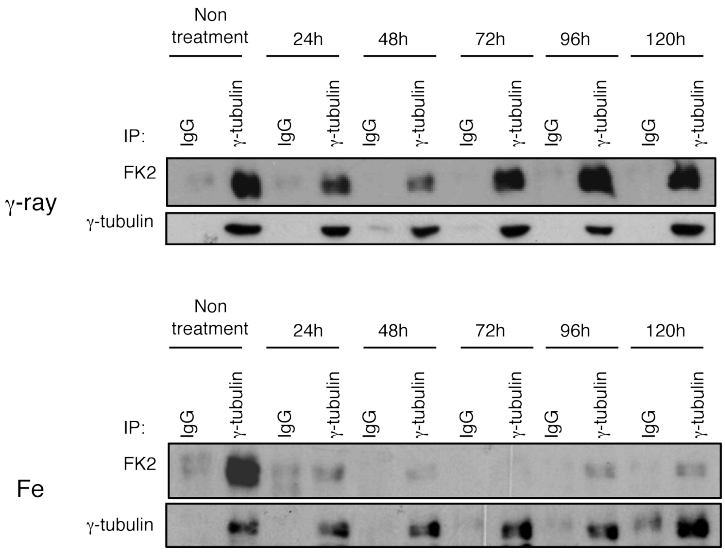


Figure 5



Supplementary figure1